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"STUDIES ON THE EFFECT OF POLYOL OSMOLYTES ON STRUCTURE, STABILITY, FUNCTIONAL ACTIVITY AND AGGREGATION PROPENSITY OF ANTIOXIDANT PROTEIN SYSTEM"

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"STUDIES ON THE EFFECT OF POLYOL OSMOLYTES ON STRUCTURE, STABILITY, FUNCTIONAL ACTIVITY AND AGGREGATION PROPENSITY OF ANTIOXIDANT PROTEIN SYSTEM"

1. Introduction:-

Many living organisms like microorganisms, animals and plants need to adapt themselves to the environment otherwise the enzymes and proteins will denature and cause the disruption of their cellular processes. Generally, these adverse environments include extremes of temperature, pH, cold stress, high levels of dietary xenobiotics, high extracellular salt, desiccation, cellular dehydration and the existence of denaturants like urea in cells. [1-3]. Such stress reactions in our body result in the generation of free radicals[4]. Continous accumulation of these free radicals above their normal physiological levels generate oxidative stress in the body which is highly adverse for cellular homeostasis and accelerates into the stochastic modification and oxidative damage to the biomolecules of the cell including proteins.[5]. Maintenance of natively folded conformation is an important determining factor for the protein stability during such oxidative stress conditions. However, native state is somewhat more stable than its denatured counterpart[6]. In fact, oxidized proteins have been shown to lose their secondary or tertiary structure and thus their stability, activity or functions are compromised.

One of the primary protein systems, expressed in response to oxidative stress includes antioxidant enzymes which mainly include Catalase, Glutathione peroxidase, Superoxide dismutase, and thioredoxins[7]. During stress an increased expression with increased activity of these enzymes has been reported under both *in vivo* and *in vitro*. In fact for performing their function, these enzymes need to retain the delicate equilibrium between functional flexibity and structural rigidity. Likewise, in a variety of oxidative stress related disorders like Alzheimer's, Parkinson's and Familial amyotrophic lateral sclerosis, these antioxidant enzymes systems are known to be perturbed[8]. Thus, an important question arises regarding the structural and functional activity regulation of these proteins under oxidative stress conditions.One of the most promising defensive strategy observed under stress conditions is the accumulation of osmolytes by majority of the organisms including bacteria, marine fishes , plants, etc[9]. Osmolytes are small molecular weight organic compounds which have been broadly divided into three classes including amino acids and their derivatives like (Histidine, glutamate, proline, etc.), Methylammonium salts (Trimethylamine-N-oxide, betaine, Glycerophosphocholine), Polyols

and sugars (Glycerol, myo-inositol, Sorbitol, Mannitol, Glucose, Glycosylglycerate etc) [10]. Among all these, polyol class has been found to be an exceptional class with wide distribution and protective effects[11]. This class mainly includes polyhydroxyl compounds like Glycerol,Sorbitol, *myo*-Inositol, Xylitol etc. Interestingly, a strong correlation has been also observed between polyols and oxidative stress disorders.

Keeping in view the ability of osmolytes to regulate cellular proteostasis under stress conditions, involvement and accumulation of various osmolytes under oxidative stress conditions and the paucity of a systematic investigation, the study was designed to investigate the effect of polyol osmolytes on structural stability, functional activity and aggregation propensity of Catalase and superoxide dismutase-an important members of cell's antioxidant system. So far no such systemic investigation has been carried out on such an important regulatory protein system. Catalases (EC1.11.1.6) are heme containing metalloenzymes present almost in all aerobically respirating organisms that play an important role in disposing off H_2O_2 produced within the cells into dioxygen and water thus protecting the cells from the toxic effects of hydrogen peroxide[12]. Similarly Superoxide dismutases are metalloproteins, containing copper and zinc, manganese or iron as the prosthetic group. Copper, zinc superoxide dismutase (EC 1.15.1.1) catalyzes a two-step dismutation of the toxic superoxide radical ($O_2^{-\cdot}$) to molecular oxygen and hydrogen peroxide through alternate reduction and oxidation of copper ion at diffusion-limited catalytic rate. Polyol Osmolytes to be involved in the study will mainly include *myo*-Inositol, Mannitol, Xylitol, Glycerol, Sucrose, sorbitol.

We have investigated the consequences of various osmolytes on activity of Catalase and SOD. It was seen that all polyol osmolytes enhanced the catalytic efficiency except myo-inositol. Myo-inositol appears to be one of the critical metabolites associated with the decline of the antioxidant potential in AD pathophysiology. In this study, we primarily attempted to investigate the functional consequences of mI on the main cytoplasmic antioxidant enzyme, catalase. We discovered that mI inhibits catalase function by increasing *K*m and altering the enzyme's overall structure and active site. The study highlights a novel role of mI in AD pathophysiology and mediating cross-talks between the cellular antioxidant enzyme systems and metabolites.

We have further elaborated the study with interested to find the effects of various other physiologically important polyol osmolytes on the function, structure and aggregation property of catalase and SOD which are physiologically and commercially an important candidate against

oxidative stress. We have observed a substantial increase in the activity of catalase in the presence of all the polyols studied by us, viz., glycerol, sucrose, xylitol and Sorbitol. This study reports the correlation of the increase in the enzymatic, structural and thermodynamic stability of enzymes in the presence of polyols with their effect on an increase in the chaperone activity (Anti-aggregation property) thus may prove as potent therapeutic agents for neurodegenerative disorders like AD, PD etc.

2. Objectives:-

- **Investigating the influence of osmolytes on functional activity of antioxidant proteins.**
- **Modulation of structural properties of anti-oxidant proteins by polyol osmolytes.**
- **Influence of polyol osmolytes on thermodynamic stability of anti-oxidant proteins at different pH values.**
- **To investigate the effects of osmolytes on aggregation propensity of antioxidant proteins.**

3. Methodology (Materials & Methods):-

A. Investigating the influence of osmolytes on functional activity of anti-oxidant proteins.

Catalase activity was based on measurement of degradation of hydrogen peroxide spectrophotometrically at 240 nm in presence and absence of osmolytes. Measurements were made spectrophotometrically according to the method of Aebi [12]. The enzyme kinetic parameters (K_m, K_{cat}, V_{max}, K_{cat} / K_m) were calculated based on Michaelis-menten and Lineweaver-Burk plot kinetics. The enzyme concentration used in the assay was 10 nM. In order to determine the effect of polyols on kinetic parameters (K_m, V_{max} and K_{cat}), the reaction was initiated by the addition of hydrogen peroxide (H_2O_2) as a substrate in the reaction mixture. Catalase mediated degradation of hydrogen peroxide was followed by monitoring the change in the absorbance at 240 nm at 25^OC for 20 min.

The activity/kinetics of Superoxide dismutase was estimated by modified method of Beauchamp and Fridovich (1971) and monitored at 560 nm by using UV-Visible Spectrophotomery. The reaction mixture consists of sodium carbonate buffer, NBT (nitro blue tetrazolium) and EDTA. The reaction is initiated by hydroxylamine hydrochloride and the change in absorbance is recorded at 560nm at 25^{o}C for 1 hr. The SOD activity is assessed by the amount of enzyme required to inhibit 50% NBT.

The initial velocity (v_o) from each progress curve of the kinetic plots of enzymes at a given substrate concentration is determined from the straight portion of first 25 seconds of the kinetic curve. The reaction fits into the first order rate kinetics in which rate of reaction is depend on the substrate concentrations. Kinetic polt of intial velocity (v_o) versus substrate (mM) is used to determine the K_m, V_{max}, K_{cat} and K_{cat} / K_m parameters by Michael menton or Lineweaver burk plots:

$$V = \frac{\mathbf{V_{max}}\,[\mathbf{S}]}{\boldsymbol{K_m} + [\boldsymbol{S}]} \longrightarrow \mathbf{1}$$

$$\frac{\mathbf{1}}{\boldsymbol{V_0}} = \frac{\mathbf{1}}{\boldsymbol{V_{max}}} + \frac{\boldsymbol{K_m}}{\boldsymbol{V_{max}}} \times \frac{\mathbf{1}}{[\boldsymbol{S}]} \longrightarrow \mathbf{2}$$

B. Modulation of structural properties of anti-oxidant proteins by polyol osmolytes.

The structural characterization of proteins in presence of the polyol osmolytes is carried out by:

- Circular Dichorism Measurements**:**

Far-UV circular Dichorism (CD) (wavelength range of 200 – 240 nm) structural studies of catalase in presence of polyol osmolytes was carried out in the (Jasco J-810 spectropolarimeter) associated with the peltier system. Pre-incubation of protein in presence of respective osmolyte at room temperature was done before taking spectra.. Spectra obtained was used to obtain fractions of α-helix (f_α), β-sheet (f_β) and unordered (f_u) conformations of the protein. The concentration of protein in the far-UV study is kept as 0.3-0.5 µM.

- UV-Vis Spectroscopy**:**

UV-Vis spectrophotometer was used to elucidate the effect of polyol osmolytes on tertiary conformation, including heme prosthetic group present within the enzyme subunits, which shows the soret absorption band near 405 nm The absorption at about 405 nm is an intrinsic probe of Catalase and is suitable for monitoring the changes within the heme environment of the enzyme. Absorption spectra of anti-oxidant proteins incubated overnight with different polyols osmolytes was recorded from 350-700 nm.

- Fluorescence measurements:

a. **Intrinsic Fluorescence** – Changes in emission spectrum of hydrophobic residues like tryptophan caused by protein conformational transitions provides the information about the three dimensional structural changes in the protein. This was done by measuring the emission spectra of proteins like catalase and SOD in presence of polyol osmolytes. The data was observed in emission wavelength range of 300-500 nm with the excitation wavelength of 280 nm.

b. **Extrinsic Fluorescence-** The surface hydrophobicity of native and myo-inositol, Mannitol, Glycerol, Xylitol, sucrose and sorbitol treated proteins is carried out by using ANS (1-Anilino-8-Naphthalene sulfonate) dye which binds to the hydrophobic patches of the proteins. In ANS binding experiments, excitation of tryptophan residues is carried out at wavelength of 360 nm, while as the emission spectra was recorded in the wavelength range of 400-600 nm. All the samples were run in triplicates. The ANS concentration was used as 16 fold to that of protein concentration. The protein concentration used in the experiment was 0.3µM and all the samples were prepared in dark to avoid photo-decomposition of ANS dye.

- **Acrylamide quenching fluorescence study:**

Quenching of Trp fluorescence in proteins by added small solutes is widely employed in structural studies, for it can provide information on the location of Trp residues with respect to the aqueous phase and on the permeability of proteins to ligands of various molecular sizes. Furthermore, quencher accessibility has been useful for revealing changes in the conformation

of the polypeptide but, more importantly, also for investigating on the nature of its dynamical structure, that is, the frequency and amplitude of structural fluctuations that permit diffusion of solutes through generally well-packed and extensively bonded internal regions of the globular fold. Intrinsic fluorescence quenching of tryptophan residues of protein by acrylamide, measured at 340 nm, was carried out by titrating with acrylamide at highest concentration of osmolyte used. The data obtained was analyzed from the gradient of the Stern-Volmer plot equation:

$$\frac{F_0}{F_i} = 1 + K_D\,[Q] \qquad \longrightarrow \quad 3$$

where F_0 and F_i represent protein fluorescence intensity in absence and presence of varying concentration of quencher. Respectively, [Q] is the concentration of quencher used, K_D is the Stern Volmer constant related to the fluorophore lifetime and the bimolecular quenching constant.

C. Influence of polyol osmolytes on thermodynamic stability of anti-oxidant proteins at different pH values.

- **<u>Thermal Denaturation Studies</u>:**

Thermal denaturation studies of the enzymes incubated in polyol osmolytes were studied in the Cary UV 100 (Agilent) spectrophotometer equipped with the peltier system. Thermal studies of the samples were done after the overnight incubation of catalase and SOD in myo-inositol (0.05, 0.15, 0.30 and 0.60 M), Mannitol (0.25, 0.5, 0.75 and 1M), Xylitol (0.25, 0.5, 0.75 and 1M), Sucrose (0.25, 0.5, 0.75 and 1M), Glycerol(10%,20%,30% and 40%) and Sorbitol (0.25, 0.5, 0.75 and 1M) concentrations. Each sample was heated from 25 to 85^{o}C and absorbance change was followed at 280 nm.The heating rate was maintained as 1^{o}C/min throughout the run. The protein concentration used is 0.2 µM (0.05mg/ml). Heat induced transition curves obtained in presence and absence of osmolytes will be analyzed for $\boldsymbol{f_D}$ (Fraction denatured) and the unfolded fraction (f_D) of the protein and was calculated by equation:

$$f_D = \frac{y - y_N}{y_D - y_N} \longrightarrow \mathbf{4}$$

$\boldsymbol{T_m}$ (midpoint of denaturation) was calculated by using a non-linear least squares analysis method according to the relation (Equation 5):

$$y(T) = \frac{y_N(T) + y_D(T)\exp[-\Delta H_m / R(1/T - 1/T_m)]}{1 + \exp[-\Delta H_m / R(1/T - 1/T_m)]} \longrightarrow \mathbf{5}$$

where $y(T)$ is the optical property at temperature T (Kelvin), $y_N(T)$ and $y_D(T)$ are the optical properties of the native and denatured protein molecules at T K, respectively, and R is the gas constant

D. To investigate the effects of osmolytes on aggregation propensity of anti-oxidant proteins.

The effect of different osmolytes on the aggregation kinetics of the protein was investigated using different probes namely light scattering kinetics, DLS studies and TEM. The aggregation kinetics will be analyzed using appropriate equations as discussed below. DLS and electron microscopy was employed to assess and analyze the aggregation species.

i. Time-dependent aggregation studies:

Light scattering aggregation studies at pH 7.4 was followed by determining the light scattering intensity i.e., the apparent absorbance at 360 nm of the sample protein solution in different polyol osmolytes. Aggregation process was followed by monitoring the change in light scattering at 360 nm for 60 minutes at 55^{o}C in the Agilent UV Cary 100 spectrophotometer. The data obtained was analyzed by the equation :

$$\boldsymbol{I = I_0 + \frac{I_f}{1 + e^{-(t - t0/b)}}} \longrightarrow \mathbf{6}$$

where "*I*" is the light scattering intensity at time ***"t",*** and **"t_0"** is the time at 50% maximal light scattering, "$\boldsymbol{I_o}$" and "$\boldsymbol{I_f}$" represent the initial base line and final plateau line, respectively. **"b"** is

a constant. Thus, the apparent rate constant , K_{app} for the formation of aggregates is given by 1/b and the lag time is given by t_0 - 2b.

ii. ***Transmission electron microscopy.*** Samples of protein in absence and presence of highest osmolyte concentration were taken on a copper grid (100 mesh × 250 μm pitch) and left at room temperature for 5 minutes. Uranyl acetate solution (1.0%) was added to samples present on the copper grid to negatively stain them. Samples were left to air dry before getting examined using FEI Tecnai G2-200kV HRTA transmission electron microscope operating at 200 kV.

Insilico-Studies

Molecular docking measurements

The crystal structure of the catalase with PDB id 1TGU was selected for the docking studies. The chain A of the crystal structure with heme moiety in the binding site was optimized using the Protein preparation wizard in Schrodinger Release 2016-1. The structure of mI (pubchem id: 892) was obtained from Pubchem Database and optimized using the ligprep module of Schrodinger. The docking studies were performed using Autodock Vina with a grid box covering the whole protein structure and the number of conformations at 100.

Molecular dynamic simulation (MDS) measurements

Simulation system: An optimized structure of the catalase chain A, with PDB id 1TGU, was used for the simulation studies. Two separate simulation systems were prepared, one without mI (in water alone) and one with mI as a co-solvent. Details of the simulation systems are summarized in Table 1. The simulation system was prepared using Chimera and simulation set-up option in Desmond. A cubic box with a 10Å buffer between the system and the edge of the box was used including periodic boundary conditions. In case of the co-solvent systems, the mI molecules, as per their molarity, were placed inside the box. The system was solvated using the

TIP3P water model and ions were added to neutralize the system. The system was relaxed using the default protocol in Desmond. Subsequently, MDS studies were carried out for 20 ns for each system in the NPT ensemble using a Nose-Hoover chain thermostat (300 K) and a Martyna-Tobias-Klein barostat (1Atm) with OPLS 2005 force field parameters.

MDS Analysis: The analysis of the three simulated trajectories were carried out using the simulation quality analysis in Maestro in the Schrodinger Release 2016-1 and VMD1.9.4. Trajectories were analyzed for total energy, Root Mean Square deviation (RMSD), Root Mean Square Fluctuation (RMSF), Radius of gyration (R_g), Number of hydrogen bonds, and Radial Distribution Function (RDF). The plots obtained were generated using Qtgrace (http://plasma-gate.weizmann.ac.il/Grace). A representative structure from the three simulations was selected based on the clustering approach using the clustering plugin in VMD 1.9.4. Based on CA atoms with a RMSD cut off of 3 Å, the last 10ns of the trajectory from each simulation were considered for clustering. The protein conformations were visualized using Chimera and the heme interaction diagram was prepared using Ligplus. The cavity volumes were measured using the CASTp program and the cartoon representations of the protein structures were generated using Chimera.

Measurement of H_2O_2 decomposition by hemin chloride

Absorption spectra of treated and untreated hemin chloride were recorded with Agilent Cary 100 UV/Vis spectrophotometer equipped with a Peltier-type temperature controller at 37˚C. The concentrations of hemin and H_2O_2 were 5 µM and 300 µM, respectively and the decomposition progress was followed at 240 nm for 30 minutes. A sample cell of 1.0 cm path length was used for all measurements.

Electron paramagnetic resonance (EPR) measurements

EPR measurements were carried out in a Bruker EMX MicroX spectrometer. Conditions used for the measurements included gain, 1×10^5; modulation amplitude, 7G; microwave power, 0.677 mW; temperature, 298K and conversion time, 40 ms. Samples with catalase concentration of 20 µM, were loaded in sealed quartz capillary tubes and transferred to the EPR cavity to obtain spectra.

Cell viability assay

For this, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was conducted as outlined by Mosmann, with slight modifications. Briefly, cells with a density of $5x10^4$cells/ml were seeded in a 96-well plate and incubated in a humidified CO_2 incubator for 24 hours to allow for cell attachment. After 24 hours of mI (200mM) treatment, the cell viability was measured. Following this, 20 µl of MTT reagent (5 mg/ml) was added to each well and incubated for 4 hours. Formazan crystals were dissolved by adding DMSO and absorbance at 570 nm was monitored by using an ELISA microplate reader.

Measurement of reactive oxygen species (ROS) generation

HeLa Cells (1×10^6 cell/ml), under standard conditions, were seeded in a 12-well plate containing media and allowed to attach for 24 hours. Following this, the cells were treated with mI (200 mM). After 24 hours of incubation, the media was removed and 25 µM of DCFDA dye was added to each well and kept in the dark for 45 minutes before fluorescence imaging by using Flow cytometry.

Cell cycle analysis

Cell cycle progression of HeLa cells was monitored by using propidium iodide (PI) dye. Analysis of cell cycle progression was performed on the basis of DNA content of cells treated with mI. After 24 hours of incubation, cells were treated with RNase A and PI dye and were kept in dark. After centrifugation, the cells obtained were analyzed for cell cycle phase distribution by using Flow cytometry.

4. Results

Chapter 1: Myoinositol System

Effect of mI on catalase activity

The activity of catalase was measured by monitoring the decrease in absorbance of H_2O_2 at 240 nm in the presence and absence of the polyol osmolytes. Kinetic curves, obtained by plotting initial velocity (V_0) versus substrate concentration, were analyzed for kinetic parameters, K_m and k_{cat} (Table 2). It was observed that in presence of increasing concentrations of mI, K_m increases and k_{cat} decreases. Furthermore, k_{cat}/K_m, a measure of catalytic efficiency, decreases from 4.38×10^7 to 1.63×10^7 S^1M^{-1} with increasing concentration of mI. In addition to this, activity of catalase was further carried out in other polyol compounds like mannitol, glycerol, and sorbitol. It was observed that, with increasing concentration of the tested polyols, K_m decreases, while k_{cat} increases at all polyol concentrations. k_{cat}/k_m was also found to increase in presence of these polyols. Altogether, activity results imply that unlike other polyols, mI reduces the activity of catalase substantially

Effect of mI on catalase structure

Secondary structure

For this, far-UV CD spectral measurements of catalase were carried out in presence of different concentrations of mI (0-0.6 M). As can be seen in Figure 1A, no significant change in the secondary structure of catalase was observed in presence of mI.

Tertiary structure

Soret absorption of catalase heme: Absorption spectra of catalase, exhibiting maximum soret absorption peak around 403 nm, was obtained in presence of different concentrations of mI. Upon addition of mI, a concentration-dependent change in heme absorption intensity with a significant red shift of about 6 nm (from 403 nm to 409 nm) was observed.

Intrinsic Fluorescence: Here, changes in the microenvironment of the tryptophan residues were monitored in presence of mI. Upon excitation at 280 nm, fluorescence emission spectra were recorded with an absorption maximum at 336 nm. In presence of mI, a concentration-dependent decrease in tryptophan fluorescence intensity with a red shift of around 8 nm was observed.

Solvent-accessible hydrophobic areas

Extrinsic fluorescence: To monitor for any change in exposure of hydrophobic patches, ANS binding experiments were carried out. With increase in mI concentration, an increase in ANS fluorescence intensity (enhanced binding) with a prominent red shift of about 11 nm was observed, which indicates increased exposure of hydrophobic residues to the solvent in presence of MI.

Compactness

Acrylamide quenching: Acrylamide quenching of fluorescence of catalase provides information about the accessibility of the quencher to the intrinsic fluorophore (trp) as well as any alteration in the microenvironment of the fluorophore. For this, quenching of trp fluorescence by

acrylamide in the presence of mI was carried out and K_D values, calculated using equation 2, were plotted (Fig. 1E). For control, the value for K_D was found to be 1.28 ± 0.04, while it was found to be 1.51 ± 0.06 in the presence of mI. Increased K_D values in the presence of mI indicate enhanced exposure of the trp residues to the surrounding aqueous environment.

Effect of mI on the conformational stability of catalase

To monitor for any mI-induced change in the overall stability of catalase, thermal denaturation studies of catalase were carried out in the presence of mI. Thermal denaturation profiles, observed in the presence and absence of mI, were analyzed by using equation 3, and the observed f_D values were plotted as a function of temperature. These plots were further analyzed for T_m by non-linear fitting using equation 4. At 0.6 M mI, a decrease of around 3.7 ^oC was observed in T_m of catalase, which suggests an overall decreased stability of catalase in the presence of mI. It was also seen that MI induces aggregation in catalase.

***In silico* studies of catalase in the presence of the mI**

Docking studies: To elucidate any possible interaction between mI and catalase, molecular docking studies were carried out. Twenty docking conformations were obtained with mI, and the docked conformations were found to occupy and cluster at different sites around the catalase. The binding affinity of mI for catalase was found to be in the range of -4.7 to -4.2 kcal/mol, and the lowest energy conformation was located near the opening of the heme binding active site channel of catalase, indicating favorable binding of mI at this position.

Molecular dynamics simulation studies: To understand the effect of mI on the overall conformation/structure of catalase, MDS studies were carried out in the presence of an mI co-solvent system, wherein the concentrations for mI ranged up to 0.6 M, with simulation in water alone serving as a control. Simulation analysis was performed on the basis of Root Mean Square

Deviation (RMSD), Radius of gyration (R_g), and Hydrogen bonds. Upon completion of simulation, MDS parameters like RMSD, R_g, RMSF, and hydrogen bond formation were estimated . In the mI co-solvent system, catalase was found to be highly dynamic and fluctuating with longer time of equilibration. With respect to RMSD of peptide backbone atoms, the values were found to decrease insignificantly for mI-bound catalase (5.13 Å) when compared to catalase alone with water system as a control (5.42 Å). However, significant changes in RMSD values were observed for the protein-heme region, with an almost two-fold increase in RMSD value in the presence of mI (0.63Å) compared to catalase alone (0.35 Å). Results obtained for heme region indicate that the heme group of catalase in the mI co-solvent system was found to undergo pronounced structural fluctuations.

Effect of mI on the hydrogen bonding pattern of catalase: Presence of hydroxyl groups in mI may allow it to interact with either water or surface protein groups. To explore the effect of mI on the interaction network of catalase, hydrogen bond interactions within the protein, and between the protein, mI, water, and the heme group were analyzed. The number of intra-protein hydrogen bonds in the presence of mI was found to be the same as that of water alone. However, in the mI system, a profound decrease in the number of hydrogen bonds between protein and water was observed, with a decrease from 526 (in water) to 389 (in mI). This loss of hydration sphere around catalase might be responsible for the decreased hydrogen bonding pattern of the catalase with water. As expected, a very small number of protein-mI hydrogen bonds were observed.

Effect on heme binding active site of catalase: Altered catalytic activity of catalase in the presence of mI encouraged us to investigate its effect on the heme binding active site cavity of catalase. In this, the representative structure of the catalase was subjected to active site cavity

measurements using CASTp program, and the identified active site cavities were visualized. An expansion of the active site cavity near the entrance of the channel, with a volume of 577 Å, was observed in the presence of mI. In addition to this, noticeable fluctuations in the threading arm (residues 1-66) and wrapping loop (residues 376-439) of catalase (Fig.3A and C) were observed. In the superposed conformation, we could see that the threading arm and the wrapping loop, in the case of the mI system (grey and red), were getting rearranged and coming close to the β-barrel part, i.e., central core of the catalase fold.

Additionally, the distance between other important channel residues, including Gln167-Leu198 and Asp127-Trp185, was also analyzed. In the presence of mI, a respective increase and decrease in the distance of Cα atoms of the Gln167-Leu198 and Asp127-Trp185, with respect to the heme center, was observed. Furthermore, mI shows binding interaction with various hydrophobic residues lining the heme active site channel, including Val125, Arg126, Trp185, Val181, His465, Trp185, and Val18.

Effect of mI on the Redox state of heme iron

To verify whether mI has any effect on the oxidation state of Fe-center of heme, H_2O_2 decomposition by hemin chloride was monitored in the presence and absence of mI, no significant change was observed in the rate of H_2O_2 degradation by hemin chloride in presence of mI.

Furthermore, EPR measurements of catalase were carried out in the presence of mI to confirm any perturbation in the redox state of catalase heme. No alteration in the redox status of the heme moiety of the protein was observed in the presence of mI. Altogether, results show that loss of catalase activity in the presence of mI is not due to any change in the redox status of heme center of catalase.

Effect of mI on cell viability, Cell Progression and ROS generation

Using MTT assay, the effect of mI on viability of HeLa cell lines was investigated. On treatment of cells with mI, a percentage decrease of about 20% was observed in the concentration of viable HeLa cells, suggesting a cytotoxic effect of mI on HeLa cells. Flow cytometry analysis showed that in the presence of mI, the cell cycle progression stopped and the cells were arrested at the G1 phase, as indicated by the increased population of cells (about 51%) in this phase. It was also observed that MI caused more production of ROS.

Chapter 2: Mannitol System

Results and Discussion

Effect of mannitol on catalase

The catalase activity measured by monitoring the reduction in absorbance of hydrogen peroxide at 240 nm in absence and presence of mannitol. The Kinetic curves which are obtained by plotting the initial velocity (V_0) versus substrate concentration were then analyzed for K_m, k_{cat} and K_{cat}/K_m parameters. The results suggested that in presence of increasing concentrations of mannitol, K_m decreases from 31.22 mM with native control and 24.53 mM with 0.70 M concentration of mannitol. The Turn over number, k_{cat} also showed the marked increase from 1.37×10^6 S^{-1} (Control) to 2.74×10^6 S^{-1} in presence of highest concentration of mannitol used. The results determine the enhancement in function of catalase in presence of varying concentrations of mannitol. For evaluating this, several structural probes (Absorption, fluorescence and Far-UV CD spectroscopic techniques) were utilized to explore the mannitol-induced structural modifications in catalase.

Effect of mannitol on the structure of catalase

Soret absorption of catalase heme: The spectra were obtained in presence of different concentrations of the mannitol. Upon addition of increasing concentrations of mannitol, concentration dependent change in absorption of heme band intensity with a significant blue shift in λ_{max} of about 2 nm (from 403 nm to 401 nm) was observed.

Intrinsic Fluorescence: In presence of increasing concentrations of mannitol, a slight increase in the tryptophan fluorescence intensity was observed. More prominently the higher concentrations observed the characteristic blue shift in the wavelength maximum of tryptophan peak. The shift of wavelength towards the lower wavelength from 336 that of control to 332 nm in presence of 0.75 M concentration of mannitol indicates the change in the polarity of protein with solvent.

Extrinsic fluorescence: With increase in mannitol concentrations, a little increase in ANS fluorescence intensity with a prominent blue shift of about 8 nm with 0.75 M mannitol concentration (reduced binding) was observed (474 nm to 466 nm). The shift of wavelength towards the lower wavelength from 336 that of control to 332 nm in presence of 0.75 M concentration of mannitol indicates reduced exposure of hydrophobic residues to the solvent thus further burying of exposed tryptophan residues to the more interior thus favors compaction of protein.

Acrylamide quenchingThe fluorescence values of F_0/F_1 were plotted against the concentrations of the acrylamide concentration used . The control indicated the value for K_{sw} to be 1.28 ± 0.04 while as it was found to be 1.15 ± 0.06 with mannitol, respectively. The decreased K_{sw} values in presence of mannitol indicate reduced exposure of the trp residues to the surrounding aqueous environment.

Effect of mannitol on secondary structure of catalase

It was observed that significant increase in secondary structure (increased CD signal at 208 and 222 nm) which is characteristic of α-helical and β-sheet structural elements was observed in presence of mannitol

Effect of osmolytes on stability of catalase

An increase of about 4.05^{o}C in T_m was observed in presence of 0.75 M mannitol concentration. Results do infer that mannitol enhanced the overall stability of the protein by stabilization of secondary and tertiary structure of catalase and anti aggregation properties were found in all concentrations of mannitol. At more pH values of 5.5 and 9.0, there is a gradual increase of T_m values determining stability at all pH values.

In silico **study of catalase in presence of the mannitol**

Docking studies: The aminoacid residues like Gln167, Lys176, Asp127 and Arg126 were involved in the interaction. Twenty docking conformations were attained with mannitol and the docked conformations were found to occupy and cluster around the catalase.The binding affinity

of mannitol for catalase was found to be in the range of -4.5 to -3.6 kcal/mol. In this case, the lowest energy conformation was situated near the opening of the heme binding active site channel of catalase.

Simulation studies:

In mannitol (green) co-solvent systems, all of the catalase amino acid residues were found to undergo with the least fluctuation . In fact, in the presence of mannitol (RMSD value of 2.68 ± 0.21), the RMSD values of protein Cα atoms were slightly lower as compared to water alone (RMSD value of 5.42 ± 0.32). Additionally, like that of RMSD of protein Cα atoms, the heme group was observed to be quite stable in presence of mannitol

Effect of osmolytes on hydrogen bonding pattern of catalase: A profound increase in the number of hydrogen bonds between protein and water was observed with an increase from 526 (in water) to 728 (in mannitol). This increase of hydration sphere around protein might be responsible for the enhanced hydrogen bonding pattern of the catalase with water.

Effect on Radial distribution function for oxygen molecules of water: The height of the first hydration peak of water at 2.8 Å, indicative of tetrahedral orientation of water molecules, was found to increase in presence of mannitol as compared water. The second and third hydration peaks (green peaks) also got enhanced in presence of highest concentration of mannitol signifying increased preferential hydration effects.

Effect on heme binding active site of catalase. Catalase undergoes conformational change around 16ns, where the flipping of the vinyl group of heme was also noticed. In the superposed conformation , Interestingly, the stabilized conformation of threading arm and the wrapping loop of catalase in the presence of mannitol is close to the crystal structure of catalase used for simulations (PDB id: 1TGU).

Aggregation kinetics using Light scattering measurements

The presence of 0.25, 0.50 and 0.75 M mannitol reduced the formation of aggregation species and almost flattened the curve and managed to keep catalase in almost natively folded state.

Chapter 3:

Glycerol, Sucrose, Sorbitol and Xylitol Catalase System

Results

Effect of osmolytes on catalase activity

The decrease in the absorbance of hydrogen peroxide determines the activity of catalase measured at 240 nm in presence and absence of the osmolytes like glycerol, sucrose, sorbitol and xylitol osmolytes. Kinetic curves obtained by plotting initial velocity versus substrate concentration were analyzed for kinetic parameters, K_m, K_{cat}, V_{max}. It was observed that in presence of increasing concentrations of glycerol, sorbitol, sucrose, xylitol and sorbitol, K_m was found to get decreased while V_{max} and K_{cat} increased in a concentration dependent manner. The catalytic efficiency K_{cat}/k_m, which determines the overall activity of enzyme, was also enhanced in presence of these polyol osmolytes. The highest increase in activity was found in presence of glycerol, where catalytic efficiency got improved from 4.379×10^4 (control) to 5.813×10^5 with 40% of glycerol.

Effect of Osmolytes on soret absorption of catalase heme

Absorption spectra of bovine liver catalase, exhibiting a maximum soret absorption peak around 403 nm, were obtained in presence of different concentrations of polyol osmolytes like glycerol, Sorbitol, sucrose and xylitol. Upon addition of these polyols, there was the concentration dependent change in heme absorption peak with a substantial blue shift of 10 nm, 3 nm and 9 nm in presence of glycerol, sorbitol and sucrose. However little change in the peak shift of 2 nm could be detected in presence of xylitol respectively. The intensity of the absorption peak got highly enhanced in presence of increasing glycerol and sucrose concentrations. The results reveal that osmolytes affected the heme micro-environment of catalase.

Intrinsic fluorescence measurements of catalase in presence of osmolytes

The structural changes observed in presence of different concentrations of glycerol, sucrose, sorbitol and xylitol were carried out by monitoring the change in the microenvironment

of the tryptophan residues. The tryptophan shows the maximum peak at 336 nm when excited at 280 nm. It was observed that in presence of glycerol, there was the slight increase in intensity with a blue shift of tryptophan peak by 4 nm (336 nm-332 nm). The increased concentration of Sorbitol, sucrose and xylitol caused decreased fluorescence intensity with blue shift of peak.

To study the changes in surface hydrophobicity of catalase molecules formed during the addition of osmolytes like glycerol, sorbitol, sucrose and xylitol (0.25,0.50, 0.75, 1.00 M, fluorescence intensity of ANS dye was observed at 480 nm. It was observed that with increasing concentrations of glycerol, sorbitol, sucrose and xylitol, a slight increase in intensity along with a prominent blue shift of emission maximum was observed. No binding of ANS was observed.

Effect of osmolytes on secondary structure

Thus to further confirm whether these polyol osmolytes have any effect on the amide bond strength of protein, Far CD spectroscopy was employed. In catalase control, far-UV CD spectrum collected at the end of run showed characteristic α-helical rich spectrum. We also observed that, in presence of different concentration of glycerol, Sorbitol, Sucrose and Xylitol (0.25-1.00 M) there was a concentration dependent increase in the secondary structure (CD Signal at 208 nm and 222 nm) of catalase.

Acrylamide quenching of catalase in presence of osmolytes:

The calculated value of strern-volmer constant, K_D of 1.28 for control and 0.62, 0.93, 0.86 and 1.21 for glycerol, sorbitol, sucrose, and xylitol. The study reveals shifting of exposed tryptophan towards the hydrophobic core of the protein. The decrease in the quenching constant also confirms the further compaction of protein.

Effect of osmolytes on thermal stability of catalase

The dependence of unfolded catalase fraction as a function of temperature with increasing concentration of polyol osmolytes. It was found that with increasing concentration of glycerol, sorbitol, sucrose and xylitol, T_m of the protein increased with an increase of 15.17^{o}C, 11.32^{o}C, 12.22^{o}C and 7.22^{o}C at highest osmolyte concentration, the highest concentration of Glycerol (40%) caused the increase of melting temperature by 15.17^{o}C, while as xylitol enhanced by 7.22^{o}C at 1M concentration used. The Tm was calculated at varying pH values of 5.5 and pH 9.0, it was found that at all pH values, osmolytes enhance the melting temperature of various degrees.

Light Scattering Experiment

We observed inhibition of aggregate formation with increasing concentration as well as the number of hydroxyl groups in polyols as observed by light scattering measurements at 360 nm at 55^{o}C. In the presence of glycerol at 0.5 to 1.00 M, however an early decrease in the scattering intensity was observed, suggesting an early delayed aggregation, followed by saturation as compared to the untreated control. The concentrations above 0.75M of glycerol even flattened the hyperbolic curve and did not let the protein to get aggregated. In the presence of increasing concentration of Sucrose, sorbitol and xylitol, a significantly reduced scattering intensity was observed.

Chapter 4:

Results

Effect of osmolytes on superoxide dismutase function

The activity of superoxide dismutase determined by the dismutation reaction of SOD on the reduction reaction of nitroblue tetrazolium. The reaction was monitored at 560 nm. The activity of SOD in absence and presence of osmolytes were observed by monitoring the change in intensity of blue colored product which absorbs at 560 nm. Kinetic curves obtained by plotting initial velocity versus substrate concentration were analyzed for relative activit). It was observed by our activity studies that polyols modulate the activity of SOD in concentration dependent manner .

Fourier-transform infrared spectroscopy and CD Spectroscopy

There is both intensity change and wavelength shifts in the amide band which determines changes in the secondary structural elements of superoxide dismutase. In CD studies, Except myo-inositol, all polyols increased the secondary structural elements of SOD. While as, MI reduced it in concentration dependant manner.

Fluorescence Studies:

The structural studies of SOD revealed that the polyol osmolytes modulates the tertiary structure of protein with varying degrees by causing the compaction of protein. While as mI has negative effect on the stability of the structure of SOD as it caused the overall conformational changes revealed by loss of secondary and tertiary structure.

Aggregartion studies

All polyols showed the remarkable anti-aggregation effect on SOD except MI which increased the hydrodynamic radii of SOD.

5. Significance of study:

Understanding how polyol osmolytes affect the stability and aggregation of anti-oxidant proteins can ultimately help in the development of potential therapeutics that can prevent or control protein aggregation during oxidative stress condition. In fact, this kind of research serves as the perfect template for the identification of novel body protein aggregation inhibitors. Furthermore, because stability is a constant problem with catalase/SOD, they hasvepromised biotechnological applications in a variety of industries, including textile, food, and others. The shelf-life enhancement of catalase and SOD by osmolytes for industrial uses will be better understood by testing osmolytes for enhancement of structural stability and functional activity.

Research Publications

1. **Ali, Fasil**, et al. "Brain Metabolite, Myo-inositol, Inhibits Catalase Activity: A Mechanism of the Distortion of the Antioxidant Defense System in Alzheimer's disease." ***ACS omega*** 7.15 (2022): 12690-12700.
   (Impact factor: 4.13)

2. **Ali, Fasil**, et al. "Effect of polyol osmolytes on the structure-function integrity and aggregation propensity of catalase: A comprehensive study based on spectroscopic and molecular dynamic simulation measurements." ***International Journal of Biological Macromolecules*** 209 (2022): 198-210.
   (Impact factor: 8.02)

3. **Ali, Fasil,** Jafar K. Lone, and K. S. Chandrashekharaiah. "Role of Mannitol in Modulating the Activity, Structure and Aggregation Propensity of Superoxide Dismutase during Various Neurodegenerative disorders." ***Annals of the Romanian Society for Cell Biology*** 25.7 (2021): 1569-1579.

4. **Ali, Fasil**, et al. " Investigating the neuroprotective role of mannitol, a novel neurometabolite accumulated during various oxidative stress disorders: Multispectroscopic and in silico approaches" ***International Journal of Biological Macromolecules"*** ***(Under Communication)*** **(Impact factor: 8.02).**

Papers presented in conferences

1. **Fasil Ali,** Jafar Khaliq Lone, K S Chandrashekharaiah, Tanveer Ali Dar "Consequences of polyol osmolytes on structural and functional stability of Catalase". Interational conference on Recent Trends in Biomedical Research organized by Indian association of biomedical scientists from 12th -14th March 2021. ***[Oral presentation]***

2. **Fasil Ali**, Jafar Khaliq Lone, K S Chandrashekharaiah, Tanveer Ali Dar. Myo-inositol demolishes the catalytic efficiency by altering the structure of catalase. International conference on "Current concepts on the role of indian medicine and phytoceuticals in maintainance of health" at Jnana Kaveri Campus, Mangalore University from 15th -17th Nov 2018. ***[Poster Presentation]***.

3. **Fasil Ali,** Jafar Khaliq Lone, K S Chandrashekharaiah, Tanveer Ali Dar. "Modification of anti-oxidant proteins during oxidative stress disorders". National Level Conference at Field marshal K M Cariappa College, Mangalore University on 5th Feb 2019. ***[Oral Presentation].***

4. **Fasil Ali,** K.S. Chandrashekharaiah, Tanveer A Dar, "Modulation of structural and functional activity of catalase by polyol osmolytes accumulated under oxidative stress disorders" in the 13th Session of JK Science Congress (JKSC 2018) ***[Oral Presentation].***

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Article

Brain Metabolite, Myo-inositol, Inhibits Catalase Activity: A Mechanism of the Distortion of the Antioxidant Defense System in Alzheimer's disease

Fasil Ali, Usma Manzoor, Reshmee Bhattacharya, Aniket Kumar Bansal, Kempohalli Sayanna Chandrashekharaiah, Laishram Rajendrakumar Singh, Suma Mohan Saraswati,* Vladimir Uversky, and Tanveer Ali Dar*

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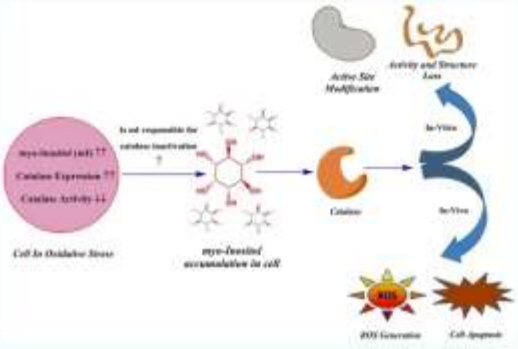
ABSTRACT: A strong correlation between brain metabolite accumulation and oxidative stress has been observed in Alzheimer's disease (AD) patients. There are two central hypotheses for this correlation: (i) coaccumulation of toxic amyloid-β and Myo-inositol (MI), a significant brain metabolite, during presymptomatic stages of AD, and (ii) enhanced expression of MI transporter in brain cells during oxidative stress-induced volume changes in the brain. Identifying specific interactive effects of MI with cellular antioxidant enzymes would represent an essential step in understanding the oxidative stress-induced AD pathogenicity. This study demonstrated that MI inhibits catalase, an essential antioxidant enzyme primarily inefficient in AD, by decreasing its k_{cat} (turnover number) and increasing K_m (Michaelis−Menten constant) values. This inhibition of catalase by MI under *in vivo* studies increased cellular H_2O_2 levels, leading to decreased cell viability. Furthermore, MI induces distortion of the active heme center with an overall loss of structure and stability of catalase. MI also alters distances of the vital active site and substrate channel residues of catalase. The present study provides evidence for the involvement of MI in the inactivation of the antioxidant defense system during oxidative stress-induced pathogenesis of AD. Regulation of MI levels, during early presymptomatic stages of AD, might serve as a potential early-on therapeutic strategy for this disease.



INTRODUCTION

The leading cause of Alzheimer's disease, the most common neurodegenerative disease in older people, has not been completely understood but is generally characterized by the deposition of extracellular β-amyloid ($A\beta$) aggregates and intracellular neurofibrillary tangles of hyper-phosphorylated tau protein.[1−4] The amyloid cascade hypothesis claims that $A\beta$ deposition drives the remaining AD pathology. Therefore, AD is detected more commonly by analyzing $A\beta$ levels in cerebrospinal fluid at the biochemical level.[5,6] Moreover, the accumulation of such toxic protein species results in increased oxidative stress, leading to synaptic dysfunction and eventual cell death.[7,8] Molecular-level understanding of the increase in oxidative stress is currently fairly low. It is well-known that despite an increase in the expression of the antioxidant enzyme system (as an attempt to suppress the increased oxidative stress) during AD pathology, the activity of the main enzyme, catalase, is drastically reduced, resulting in increased oxidative stress.[9] One central mechanism for the reduced catalase activity is believed to be the direct interaction of $A\beta$ with antioxidant enzymes, which inhibits antioxidant functions.[10] Thus, post-translational events (rather than the transcriptional processes) are believed to result in catalase inactivation, leading to impairment in the intracellular hydrogen peroxide (H_2O_2) degradation. Furthermore, AD pathophysiological conditions also involve specific alterations in the neuronal metabolites, including Myo-inositol (MI), choline, glutamate, N-acetyl-aspartate, and sorbitol.[11,12] Nevertheless, the molecular mechanisms underlying the altered metabolite levels and the inactivation of the antioxidant mechanism remain largely unexplored.

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Effect of polyol osmolytes on the structure-function integrity and aggregation propensity of catalase: A comprehensive study based on spectroscopic and molecular dynamic simulation measurements

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ABSTRACT

Owing to the ability of catalase to function under oxidative stress vis-à-vis its industrial importance, the structure-function integrity of the enzyme is of prime concern. In the present study, polyols (glycerol, sorbitol, sucrose, xylitol), were evaluated for their ability to modulate structure, activity and aggregation of catalase using *in vitro* and *in silico* approaches. All polyols were found to increase catalase activity by decreasing K_m and increasing V_{max} resulting in enhanced catalytic efficiency (k_{cat}/K_m) of the enzyme. Glycerol was found to be the most efficient polyol with a k_{cat}/K_m increase from 4.38×10^4 mM^{-1} S^{-1} (control) to 5.8×10^5 mM^{-1} S^{-1}. Correlatively with this, enhanced secondary structure with reduced hydrophobic exposure was observed in all polyols. Furthermore, increased stability, with an increase in melting temperature by 15.2 °C, and almost no aggregation was observed in glycerol. Overall, ability to regulate structure-function integrity and aggregation propensity was highest for glycerol and lowest for xylitol. Simulation studies were performed involving structural dynamics measurement, principal component analysis and free energy landscape analysis. Altogether, all polyols were stabilizing in nature and glycerol, in particular, has potential to efficiently prevent not only the aggregation of the antioxidant defense system but might also serve as a stability aid during industrial processing of catalase.

1. Introduction

The maintenance of a proper folded state (Native state) is an important determining factor for protein function. Indeed, protein stability is directly related to the enzyme function as it determines the amount of functionally active fractions [1–3]. Owing to this, maintaining protein stability has become, not only important, but an unavoidable issue for proteins employed in biotechnological, biomedical and pharmaceutical sectors [4–6]. Among other strategies like genetic engineering, immobilization etc. [7–9], one of the most popular and effective ways of protein stabilization involves the use of small molecular weight organic molecules, named as osmolytes, which always favor the folded state of proteins [10,11]. These osmolyte co-solvents mainly include sugars, polyols, amino acids and methylamines. Although being a routine strategy for stability improvement, the mechanism by which these osmolytes stabilize native state is still contentious. Among different classes of osmolytes, polyols are observed to be one of the most effective protein stabilizers with a capability of raising the thermostability of proteins as much as by 15 °C [12–14]. Moreover, it has been reported that polyols have unique ability to refold proteins as compared to other osmolyte classes [15]. Similar other studies concluded polyols to act as exceptional stabilizers [1,16–18]. Among polyols, the effect of xylitol on catalase has been only studied and compared to the other osmolyte classes (compatible and non-compatible) [19].

Catalase, a heme containing oxidoreductase enzyme, catalyzes breakdown of hydrogen peroxide to water and molecular oxygen. Under

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Role of Mannitol in Modulating the Activity, Structure and Aggregation Propensity of Superoxide Dismutase during Various Neurodegenerative disorders

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Abstract

The accumulation of small organic molecules and other low molecular weight metabolites is a widespread response that may protect diverse range of organisms against the environmental stress conditions. Accumulation of high concentrations of these organic metabolites, organisms adapt to the environmental perturbations that may lead to the structural alterations in their cellular proteins. Mannitol is one the main organic osmolyte synthesized in huge amounts as a stress-responsive factor especially in brain cells to retain cellular integrity when cells are exposed to environmental stresses. We intended to investigate the effect of mannitol on functional, structural stability and aggregation pattern of superoxide dismutase, which plays a key role against oxidative stress. We observed that mannitol increased the activity of superoxide dismutase several folds by stabilizing the secondary and tertiary structure of SOD. In addition to this, mannitol inhibited the formation of fibrils. Higher concentrations significantly decreased the formation of high molecular weight oligomers. Thus, Mannitol could be a promising scaffold for the development of therapeutic agent in a variety of neurodegenerative disorders.

Key Words: Mannitol, Oxidative stress, Superoxide Dismutase, Aggregation, Amyloid.

Introduction

Oxidative stress is linked to a number of degenerative pathways that contribute to cellular dysfunction and eventually cellular death[1]. Cells defend the oxidative stress by production and accumulation of various small organic molecules to protect the macromolecular structure under these stressful conditions[2]. Such organisms undergoing stresses accumulate higher concentrations of these molecules especially polyhydroxy compounds (up to 400 mM) in their cellular tissues during oxidative stress disorders[3]. Mannitol is one of the important metabolite that has been found to get accumulated in multiple oxidative stress disorders especially neurodegenerative diseases[4]. The prime and most vulnerable target for oxidative stress induced

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Declaration:

This is to certify that the work in this report "Studies on the effect of polyol osmolytes on structure, stability, functional activity and aggregation propensity of antioxidant protein system" has been carried out by Fasil Ali, under the supervision of Prof. K. S. Chandrashekharaiah (Deptt. of Biochemistry, Jnana Kaveri Campus, Mangalore University). It is further certified that no research work under this heading has previously been submitted to the Mangalore University or any other institution for any degree, to the best of our belief.

Ph.D Scholar **Research Supervisor**

Chairman
DOS in Biochemistry

Dated : 15th Dec 2022